

SUPPORTING INFORMATION:

Single Vehicular Delivery of siRNA and Small Molecules to Control Stem Cell Differentiation

Shreyas Shah[†], Aniruddh Solanki[†], Pijus K. Sasmal and Ki-Bum Lee*

Department of Chemistry & Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, NJ, USA

Correspondence:

Prof. Ki-Bum Lee

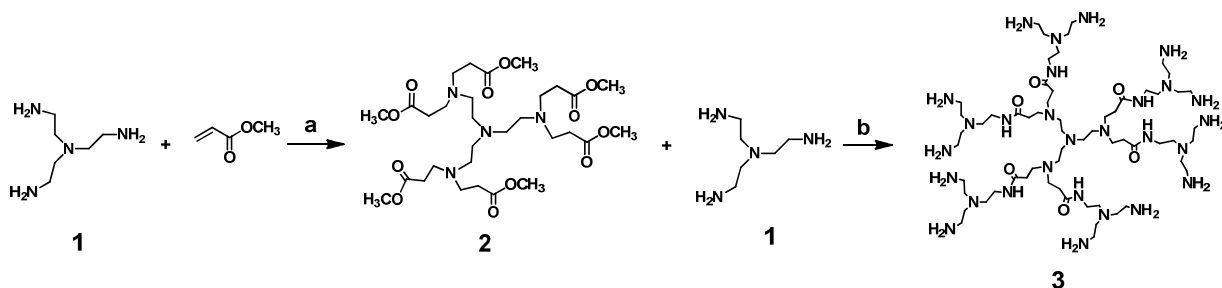
Department of Chemistry and Chemical Biology
Rutgers, The State University of New Jersey
Piscataway, NJ 08854 (USA)
Email: kblee@rutgers.edu
<http://rutchem.rutgers.edu/~kbleeweb/>

GENERAL

β -cyclodextrin, tosylimidazole, tris(aminoethyl)amine, methyl acrylate, amberlite IRA 900 were obtained from Sigma-Aldrich and used as received unless otherwise noted. Other chemicals and solvents were of analytical reagent grade. All reactions were conducted in flame-dried glassware with magnetic stirring under an atmosphere of dry nitrogen. Reaction progress was monitored by analytical thin layer chromatography (TLC) using 250 μ m silica gel plates (Dynamic Absorbents F-254). Visualization was accomplished with UV light and potassium permanganate stain, followed by heating. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on either a Varian-300 instrument (300 MHz), Varian-400 instrument (400 MHz) or a Varian-500 instrument (500 MHz). Chemical shifts of the compounds are reported in ppm relative to tetramethylsilane (TMS) as the internal standard. Data are reported as follows: chemical shift, integration, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, br=broad, m=multiplet), and coupling constants (Hz).

METHODS

1. Synthesis of DexAM



Scheme 1. First Round of Micheal Addition and Amidation, a) MeOH, 0°C, 1h/ RT, 48h, b) MeOH, 0°C, 1 h, RT, 7 days

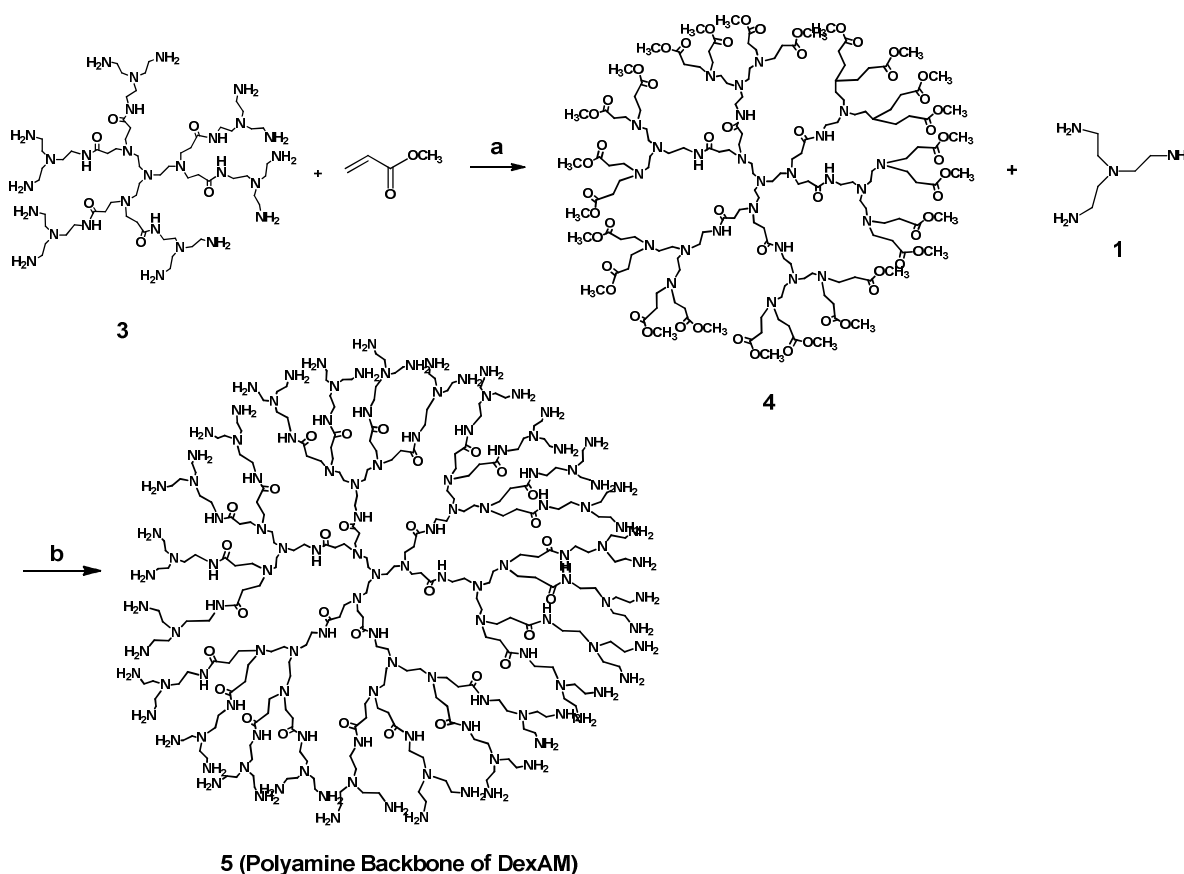
1.1 Synthesis of hexamethyl-3,3',3'',3''',3''''-(2,2',2''-nitriлотris(ethane-2,1-diyl)tris(azanetriyl)) hexapropanoate (2)

A solution of tris(aminoethyl)amine (**1**, 4.3872g, 30 mmol) in methanol (25 mL) was added dropwise to a stirred solution of methyl acrylate (19.37 g, 225 mmol) in methanol (25 mL) for 1 h in an ice-water bath. The resulting solution was stirred for 1 h in an ice-water bath and then allowed to warm to room temperature and stirred for further 48 h. The solvent and excess acrylate were removed under reduced pressure using a rotary evaporator. The residue was purified by column chromatography to afford the product (**2**) as a colorless oil. Yield: 16.88 g, 85%).; NMR (300 MHz, CDCl₃): δ 2.44 (t, J=6.9 Hz, 12H), 2.49 (s, J=6 Hz, 12H), 2.74 (t, J=6.9 Hz, 12H), 3.67 (s, 18H). MS (m/z): calculated, 662.37 for C₃₀H₅₄N₄O₁₂; found, 685.76 for [M + Na]⁺.

1.2 3,3',3'',3''',3''''-(2,2',2''-nitriлотris(ethane-2,1-diyl)tris(azanetriyl))hexakis(N-(2-(bis(2-aminoethyl)amino)ethyl)propanamide) (3)

A solution of **2** (2.17 g, 3.3 mmol) in methanol (20 mL) was added dropwise to solution of tris(aminoethyl)amine (**1**, 5.8 g, 39.6 mol) in methanol (20 mL) and stirred over a period of 1 h in an ice bath. The resulting solution was allowed to warm to room temperature and stirred for 7 days at room temperature at which time no methyl ester was detectable by NMR spectroscopy. The solvent was removed under reduced pressure using a rotary evaporator and then the excess tris(aminoethyl)amine was removed using an azeotropic mixture of toluene and methanol (90:10 v/v). The remaining toluene was removed by azeotropic distillation using methanol. Finally, the remaining methanol was removed under vacuum. The residue was purified by dialysis and centrifugal filtration to afford the desired product. Finally the product was

kept under vacuum to obtain the amino-terminated product (**3**, 4.4 g, 99%) as colorless oil. NMR (300 MHz, CDCl₃): δ 1.25 (s, J=6.0 Hz, 24H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.25 (t, 12H), 8.0 (s, 6H). MS (m/z): calculated, 1347.14 for C₆₀H₁₃₈N₂₈O₆; found, 1370.0391 for [M + Na]⁺.



Scheme 2. Second Round of Micheal Addition and Amidation, a) MeOH, 0°C, 1 h, RT, 60 h, b) MeOH, 0°C, 1 h, RT, 7 days

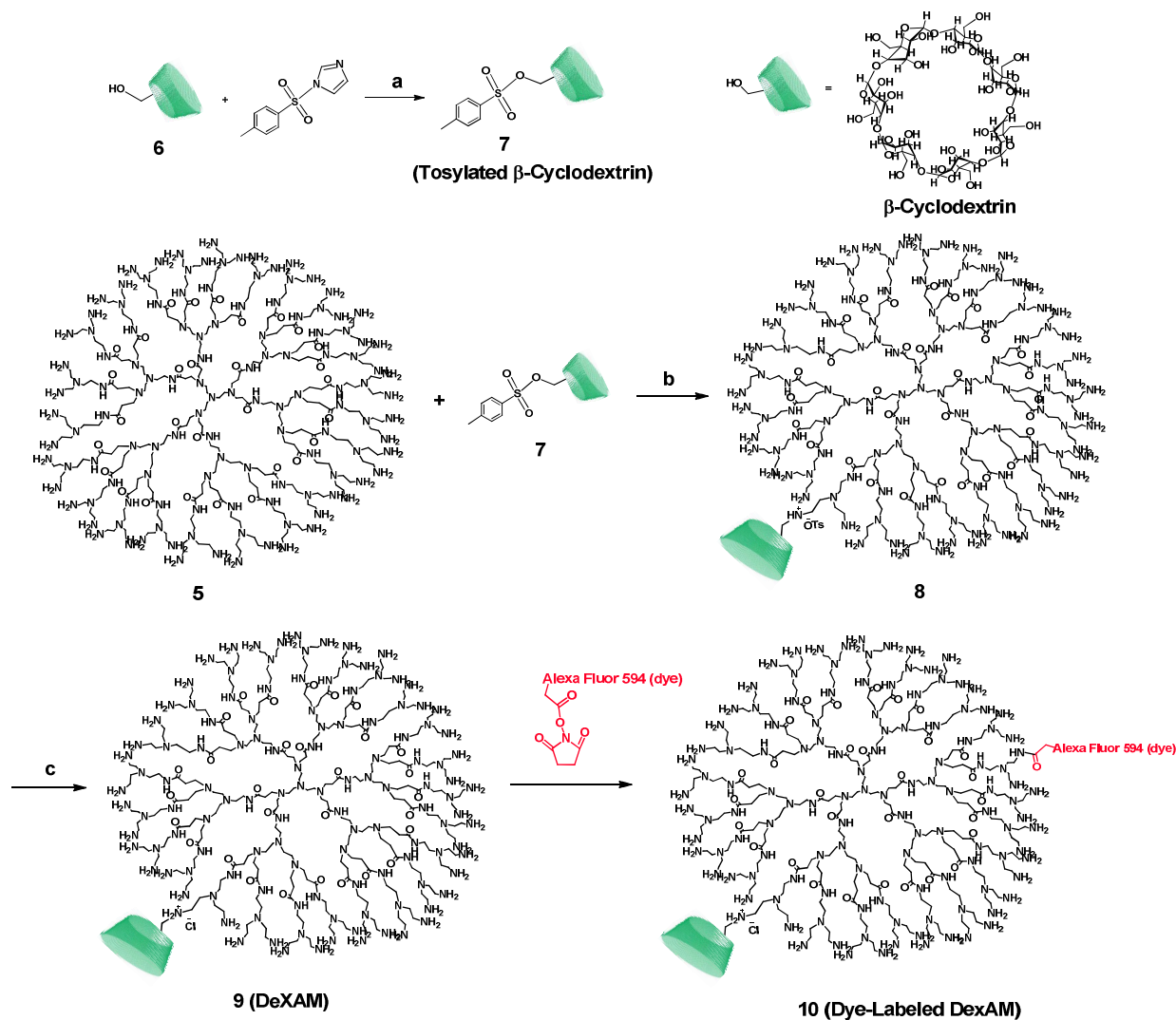
1.3 Synthesis of methyl ester of **3** (**4**)

A solution of **3** (1.48g, 1.1 mmol) in methanol (5 mL) was added dropwise to a stirred solution of methyl acrylate (2.84 g, 33.0 mmol) in methanol (5 mL) for 1 h in an ice bath. The resulting solution was stirred for 30 min in an ice bath and then for 60 h at room temperature. The volatiles were removed under reduced pressure. The residue was purified by column chromatography using DCM:MeOH (10:1 v/v) to afford the desired product (**4**) as a yellow oil. Yield: (3.41 g, 91%); NMR (300 MHz, CDCl₃): δ 2.44 (t, J=6.9 Hz, 12H), 2.49 (s, J=6 Hz, 12H), 2.74 (t, J=6.9 Hz, 12H), 3.67 (s, 18H). MS (m/z): calculated, 3410.03 for C₁₅₈H₂₈₄N₂₆O₅₄; found, 3435.08 for [M + Na]⁺.

1.4 Synthesis of the polyamine backbone of DexAM (**5**)

A solution of ester **4** (3.41g, 1 mmol) in methanol (20 mL) was added dropwise to a stirred solution of tris(aminoethyl)amine (**1**, 7.02 g, 48 mmol) in methanol (20 mL) over a period of 1 h

in an ice bath. The resulting solution was allowed to warm to room temperature and stirred for 7 days at room temperature at which time no methyl ester was detectable by NMR spectroscopy. The solvent was removed under reduced pressure the excess tris(aminoethyl)amine was removed using an azeotropic mixture of toluene:MeOH (90:10 v/v). The product was further purified by washing with anhydrous ether twice, yielding a highly viscous liquid. Finally the product was kept under vacuum to provide the amino-terminated final product (**5**) as a light yellow liquid. Yield (6.1 g, 99%); NMR (300 MHz, CDCl₃): δ 1.25 (s, J=6.0 Hz, 24H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.25 (m, 12H), 8.0 (s, 6H). MS (m/z): calculated, 6151.06 for C₂₇₆H₆₁₈N₁₂₄O₃₀; found, 6177.66 for [M + Na]⁺.



Scheme 3. Conjugation of β -CD to the Polyamine Backbone, a) ddH₂O, RT, 4h / 1 % NaOH, 10 min/NH₄Cl, b) DMF, 90°C, 48h, c) Amberlite IRA900

1.5 Synthesis of mono-tosylated cyclodextrin (**7**)

β -cyclodextrin (**6**, 8.75 g, 7.71 mmol) and tosylimidazole (2.22g, 10.0 mmol) was dissolved in 88 ml deionized water. The solution was vigorously stirred for 4 h at room temperature. Aqueous NaOH solution (1% (w/v), 10.0 ml) was gradually added to the solution and stirred for an additional 10 min. The insoluble solid was filtered off and the filtrate was collected. The filtrate was neutralized to pH 7 using NH₄Cl to induce precipitation. The precipitate was then collected by filtration, washed with cold water (25 ml x3) and with acetone (25 ml x4). The solid was dried in a drying oven at 60 °C under vacuum (10 mm Hg) overnight to yield **7** as a white solid (4.5 g,

51% yield). ^1H NMR (300 MHz, DMSO- d_6), δ 7.72 (d, $J=8.4$ Hz, 2H), 7.41 (d, $J = 8.4$ Hz, 2H), 5.60–5.89 (m, 14H), 4.75–4.81 (m, 7H), 4.15–4.62 (m, 6H), 3.45–3.72 (m, 28H), 3.15–3.47 (m, 24H), 2.41 (s, 3H). MS (m/z): calculated, 1,288.4 for $\text{C}_{49}\text{H}_{76}\text{O}_{37}\text{S}$; found, 1,311.5 for $[\text{M} + \text{Na}]^+$.

1.6 Synthesis of water-soluble CD-Polyamine tosylate (8)

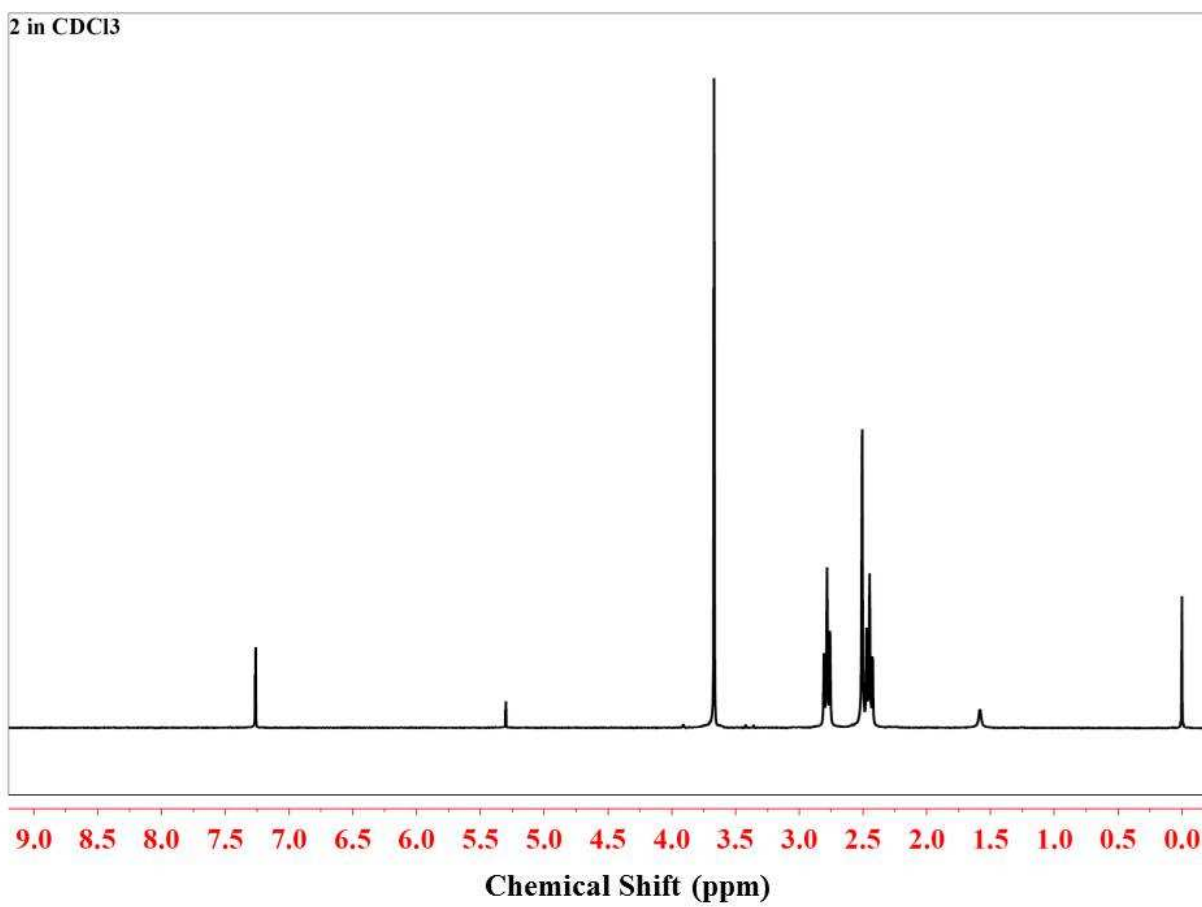
Tosylated β -CD (**7**, 0.98 g, 0.75 mmol) and polyamine (**5**, 1.53 g, 0.25 mmol) were dissolved in DMF (10 mL) in a 25 mL one-necked round-bottomed flask equipped with Liebig's condenser and a stir bar. The flask was degassed and purged with nitrogen. The mixture was stirred and heated at 90 °C for 48 h. The reaction mixture was cooled down to room temperature and product precipitated out by the addition of acetone (20 mL). The precipitate was collected by filtration, washed with acetone and dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **8** as a brown solid. Yield: (2.23 g, 89%); ^1H NMR (300 MHz, DMSO- d_6), δ 1.25 (s, $J=6.0$ Hz, 24H), 2.41 (s, 3H), 2.44 (t, $J=6.9$ Hz, 12H), 2.48 (m, 72H), 2.74 (t, $J=6.9$ Hz, 12H), 3.15–3.47 (m, 24H), 3.25 (t, 12H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.21 (d, $J = 8.4$ Hz, 2H), 7.52 (d, $J=8.4$ Hz, 2H), 8.0 (s, 6H).

1.7 Anion exchange reaction for obtaining DexAM (9)

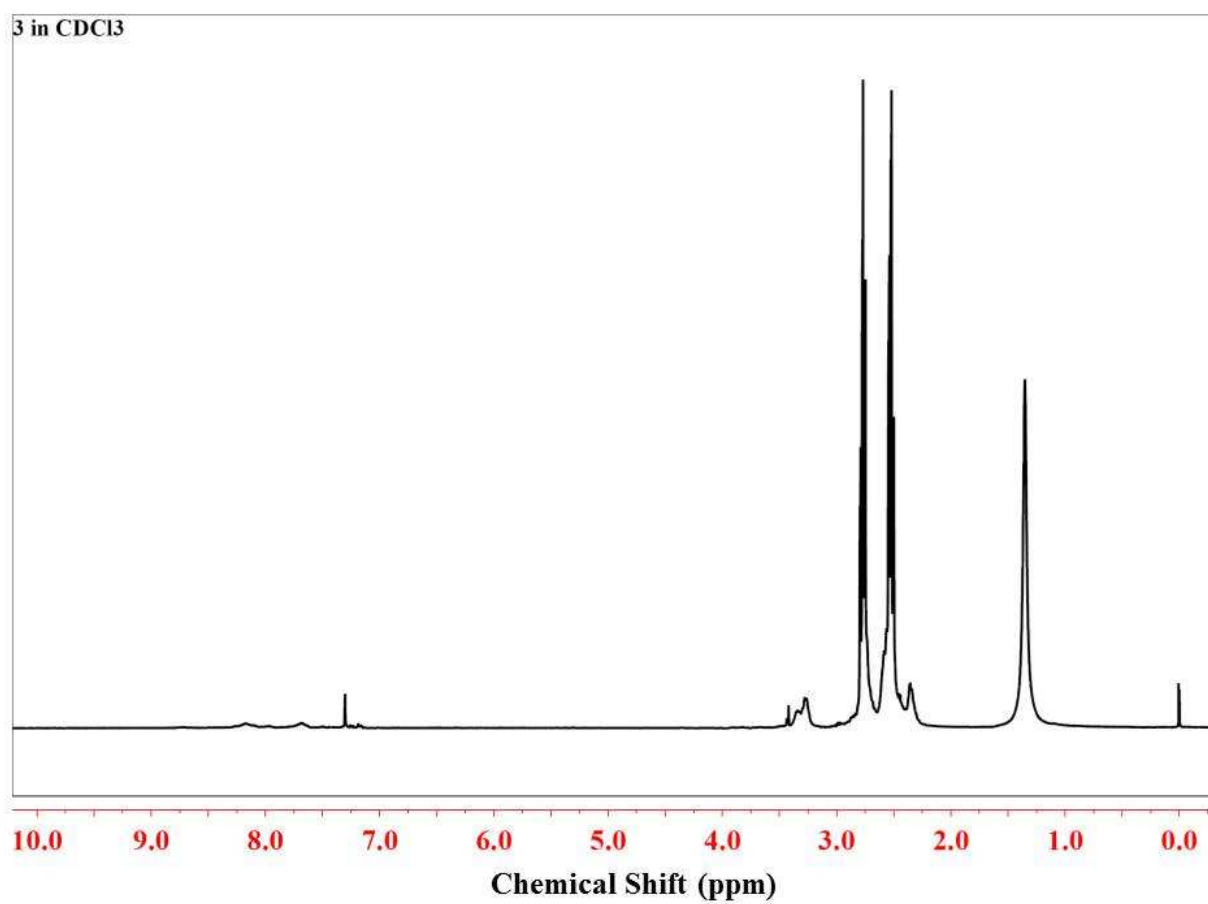
CD-polyamine tosylate (**8**, 5.03 g, 0.5 mmol) was dissolved in 40 mL deionized water. A 50 mL solid-phase synthesis vessel was packed with Amberlite IRA-900 ion-exchange resin to about half the vessel volume. The solution was transferred into the solid phase vessel. After 1 h, the eluent was collected and the water was distilled off under reduced pressure using a vacuum pump. The solid residue was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **9** as a brown solid. Yield: (4.58 g, 95%); ^1H NMR (300 MHz, D_2O): δ (ppm) 5.07 (H1, β -CD), 4.12–3.73 (H3, H5, H6, β -CD), 3.71–3.53 (H2, H4, β -CD), 3.50–3.13 (dendrimer methylene), 3.05–2.53 (dendrimer methylene), 2.51–2.11 (dendrimer methylene).

1.8 Conjugation of Alexa-Fluor 594 dye to DexAM (10)

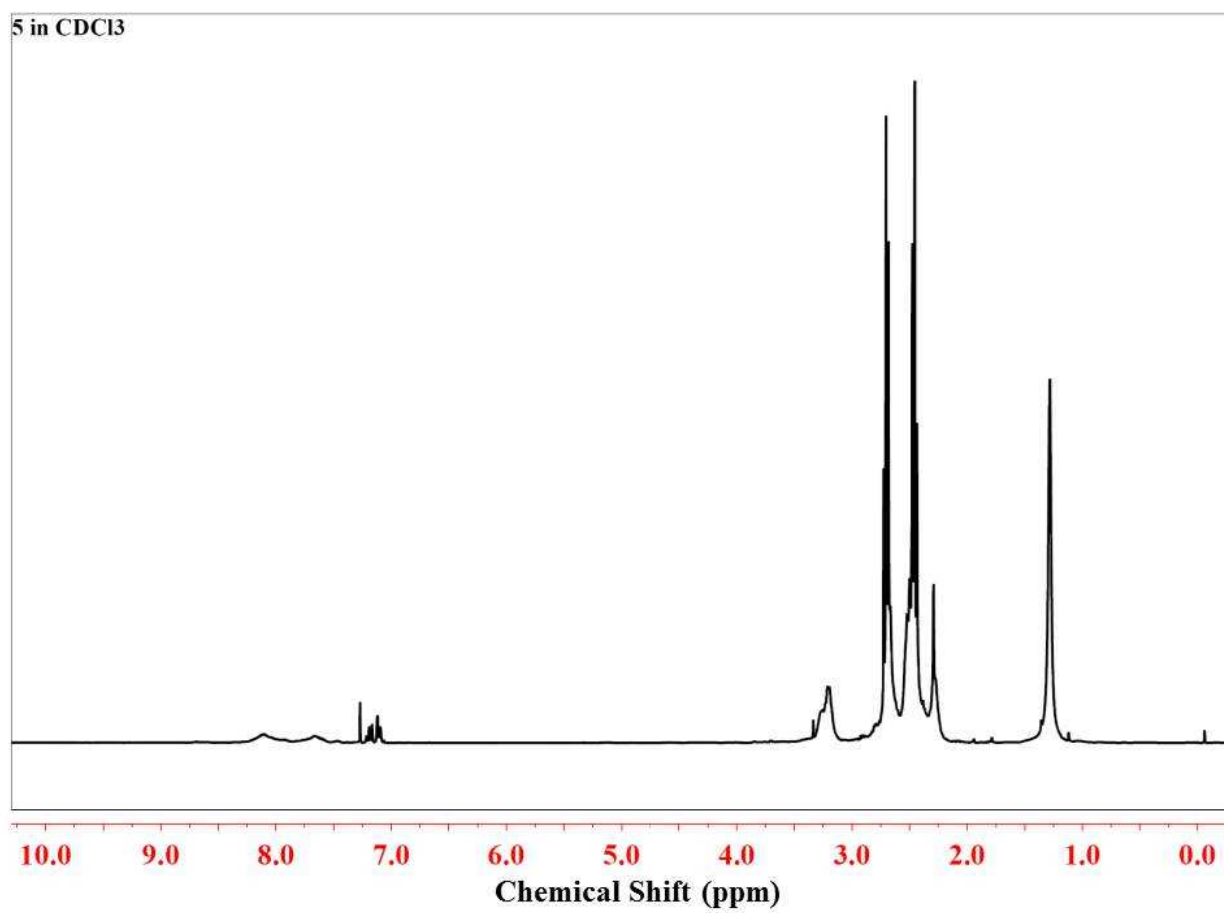
Alexa Fluor-594 dye (100 nM, Molecular Probes) and DexAM (**9**, 100 nM) were dissolved in PBS buffer solution (0.5 mL). The reaction mixture was allowed to vortex for 5 minutes. After being vortexed, the mixture was shaken at room temperature for 3 h. To ensure the removal of any excess dye, the sample was purified using centrifugal filtration with a 3000 MWCO filter (Millipore).



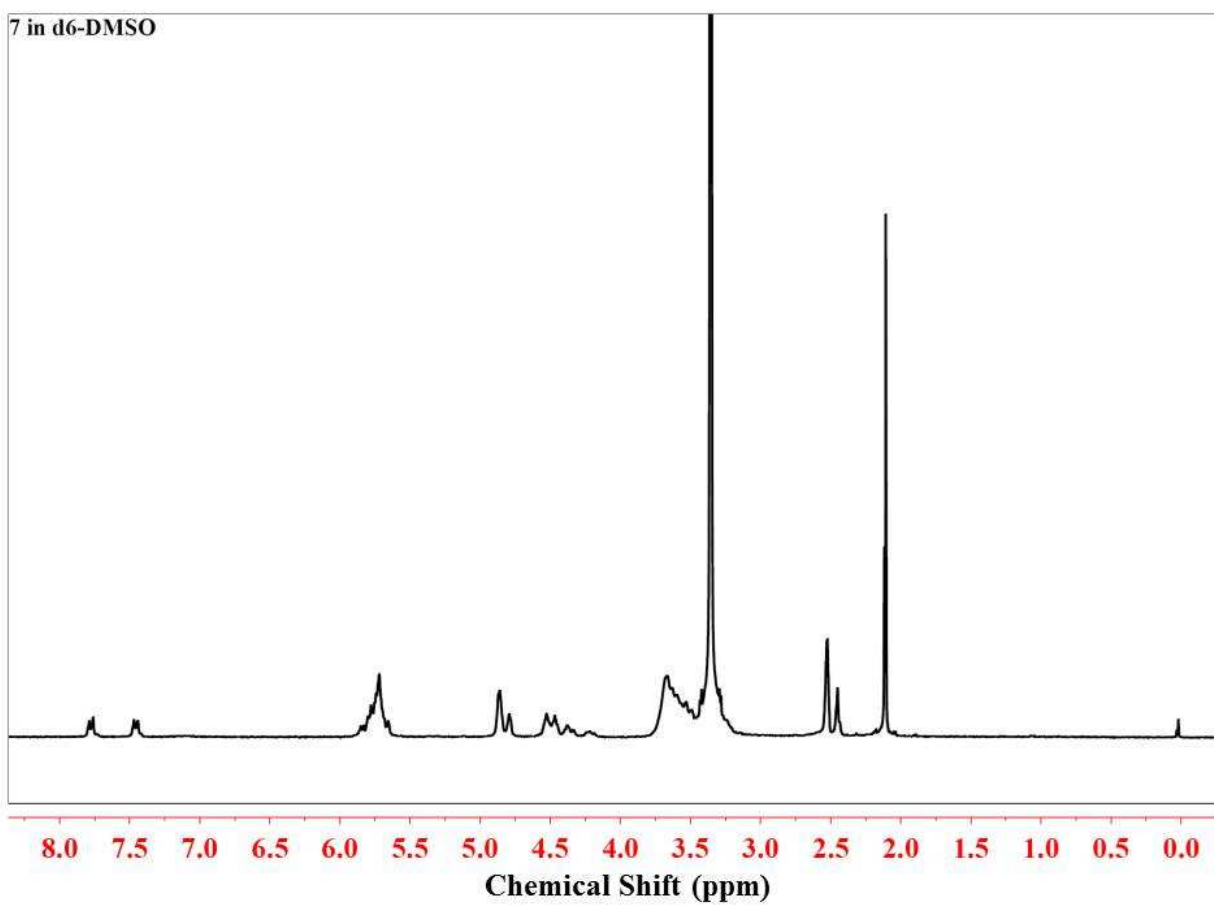
¹H NMR of compound **2** in CDCl₃



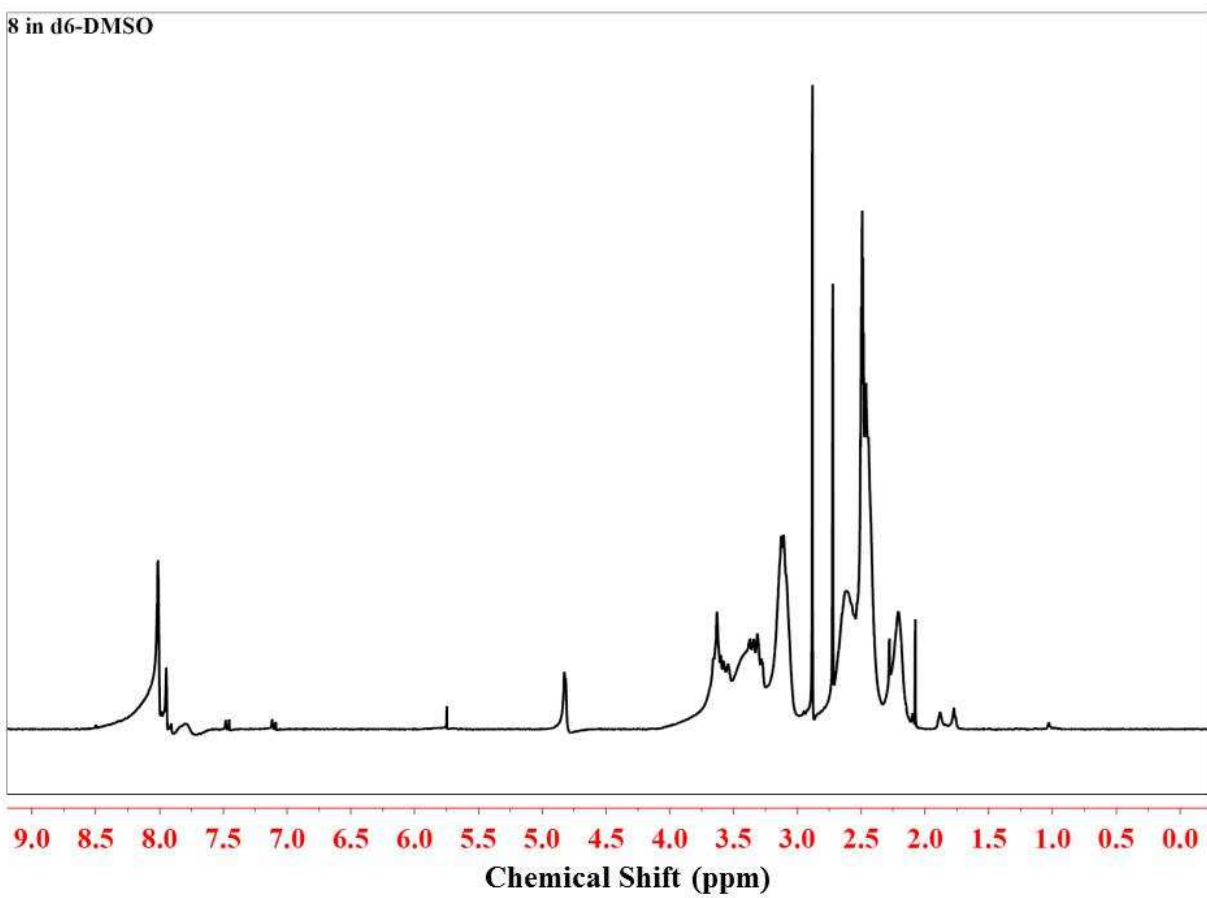
¹H NMR of compound **3** in CDCl₃



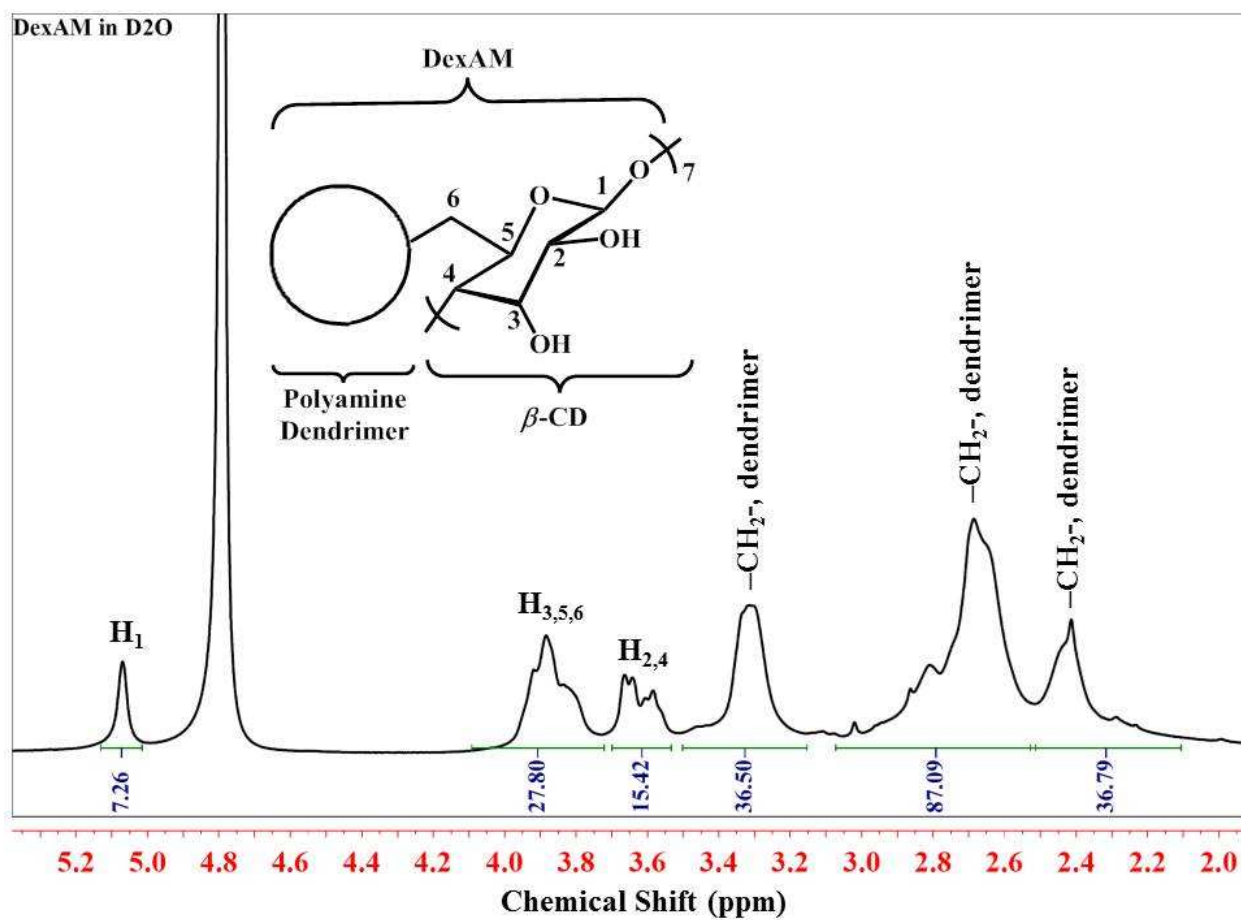
¹H NMR of compound **5** in CDCl₃



^1H NMR of compound **7** in $\text{d}_6\text{-DMSO}$

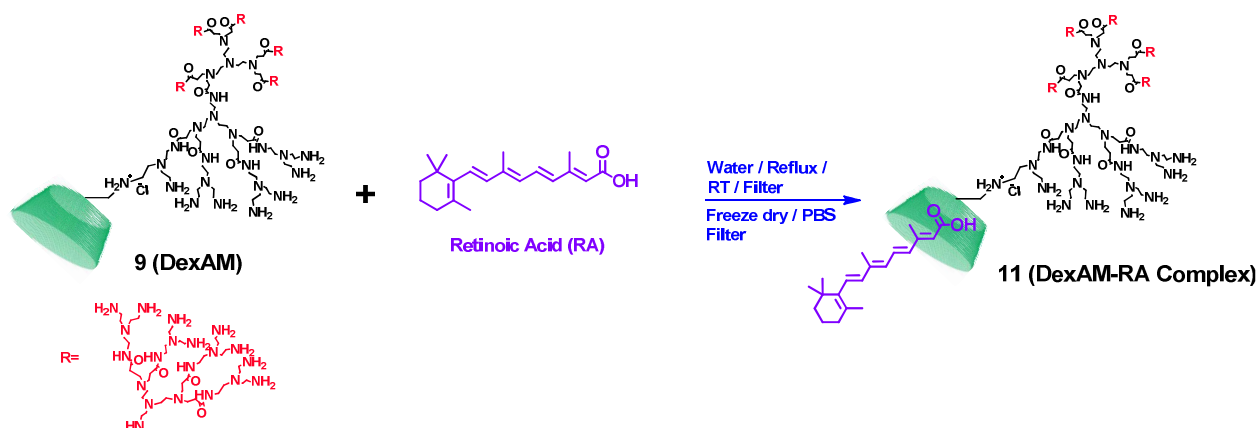


^1H NMR of compound **8** in $\text{d}_6\text{-DMSO}$



^1H NMR of compound **9** in D₂O

2. Inclusion of RA into DexAM complex



Scheme 4. Inclusion of RA into DexAM

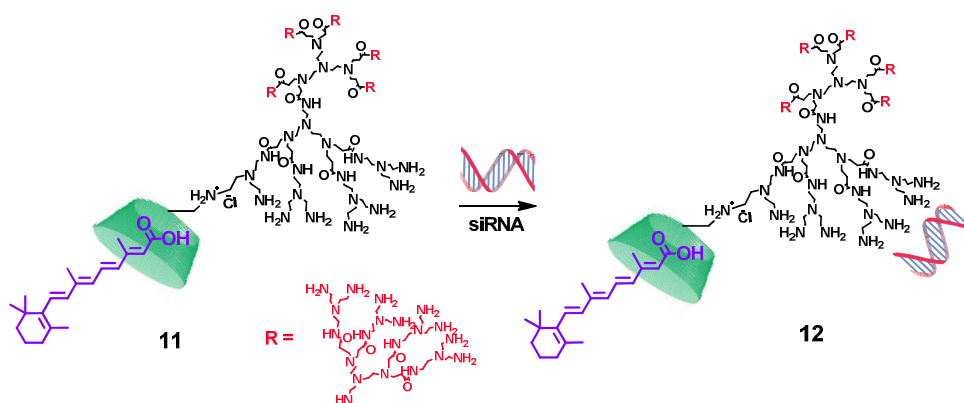
Procedure for RA inclusion in DexAM

3 molar equivalent of Retinoic acid (RA) was solubilized in 1 molar equivalent of DexAM in distilled water. Briefly, 11.5 mg of RA was added to a solution of 123 mg of DexAM in 3 mL of water, heated and refluxed overnight, and then rapidly cooled to room temperature. The reaction mixture turned to dark brown color after overnight reflux. This solution was filtered and freeze-dried. Thereafter, 200 μ L PBS was added to DexAM complex (10 μ mol) and allowed to stand at room temperature to dissolve for a few minutes. Finally, the complex was purified through by centrifugal filtration using a 3000 MWCO membrane (Millipore). DexAM-RA solutions of various concentrations were prepared by maintaining the molar ratio between RA and DexAMs.

Measuring RA Encapsulation Efficiency

A suspension of DexAM (40 mg) and varying mole equivalents of RA was refluxed in distilled water. After cooling to room temperature, the reaction mixture is diluted with excess water and extracted with dichloromethane to isolate free RA. The organic phase was washed with brine, dried over sodium sulfate and concentrated *in vacuo*. The amount of RA was measured using UV-visible spectroscopy and each reaction was carried out twice. **Figure S6** displays the percent RA encapsulation per β -CD on the DexAM versus the mole equivalent of RA.

3. Formation of Polyplexes between siRNA and DexAM



Scheme 5. Polyplex formation of DexAM and siRNA.

siRNA complexation: DexAM/siRNA complexes were prepared by adding equal volumes of DexAM solution (DexAM dissolved in water) and siRNA (dissolved in water) at varying different concentrations in culture media. The resultant solution was then incubated for 30 min at room temperature before transfection. The GFP siRNA sequence was: Antisense – 5'-CCAACGACAUCAGCGACUAUU-3', Sense – 3'-UUGGUUGCUGUAGUCGCUGAU-5'. The SOX9 siRNA sequence was Antisense – 5'-AACGAGAGCGAGAAGAGACCC-3', Sense – 3'-TTGCUCUCGCUCUUCUCUGGG-5'.

4. Rat Neural Stem Cell (NSC) Culture, Cell Viability Assay & Immunocytochemistry

4.1 NSC Culture and Differentiation

Rat neural stem cell line (Millipore) were purchased and routinely expanded according to the manufacture's protocol. The NSCs were maintained in laminin (Sigma, 20 µg/ml) coated culture dishes precoated with poly-L-lysine (10 µg/ml) in Millitrac media (Millipore) supplemented with the antibiotics, penicillin and streptomycin (Life Technologies), in the presence of basic fibroblast growth factor (bFGF-2, 20 ng/ml, Millipore). All of the cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. For consistency, the experiments were carried out on cells between passages 2 and 5. Neural differentiation was initiated by changing the medium to basal medium (without bFGF-2). The cells were allowed to differentiate for 6 days with the basal medium in each being exchanged every other day.

4.2 Cell Viability Assay

The percentage of viable cells was determined by MTS assay following standard protocols described by the manufacturer. All experiments were conducted in triplicate and averaged. The quantification of cytotoxicity was done using MTS assay after incubating cells in the presence of the DexAM-siRNA complexes.

4.3 Immunocytochemistry

To investigate the extent of neuronal differentiation, at Day 6, the basal medium was removed and the cells fixed for 15 minutes in Formalin solution (Sigma) followed by two PBS washes. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and non-specific binding was blocked with 5% normal goat serum (NGS, Life Technologies) in PBS for 1 hour at room temperature. To study the extent of neuronal differentiation the primary mouse antibody against TuJ1 (1:500, Covance) and primary rabbit antibody against MAP2 (1:100, Cell Signaling) was used and for glial differentiation the primary rabbit antibody against GFAP (1:300, Dako) was used. The fixed samples were incubated overnight at 4°C in solutions of primary antibodies in PBS containing 10% NGS. After washing three times with PBS, the samples were incubated for 1 h at room temperature in solution of anti-mouse secondary antibody labeled with Alexa-Fluor® 647 and anti-rabbit secondary antibody labeled with Alexa-Fluor® 546 (1:200, Life Technologies), Hoechst 33342 (1:500, Life Technologies) in PBS containing 10% NGS to observe neuronal and glial differentiation. After washing the cells thrice with PBS, they were imaged using Nikon TE2000 Fluorescence Microscope. ImageJ (NIH) was used for comparative analysis and quantifying the cells expression TuJ1 and GFAP.

SUPPORTING FIGURES	
Figure S1	siRNA complexation ability of DexAMs using Picogreen assay
Figure S2	Dye-labeled siRNA uptake in NSC
Figure S3	Quantitative comparison of GFP Knockdown using commercial transfection reagents
Figure S4	Cell viability of NSCs treated with DexAM
Figure S5	Comparison of NSC viability using commercially available transfection reagents
Figure S6	Percentage of RA encapsulation per β -CD on the DexAM
Figure S7	Quantitative comparison of NSC differentiation for various control experiments

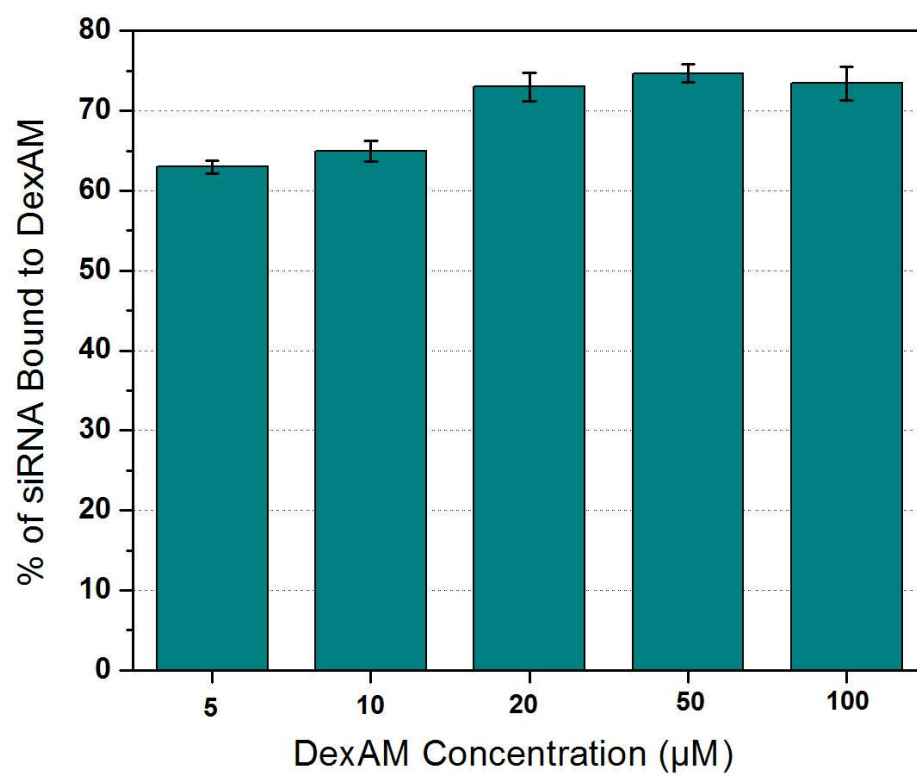


Figure S1. siRNA complexation efficiency of DexAM measured by Picogreen dye exclusion assay (Absorption = 480 nm, Emission = 520 nm).

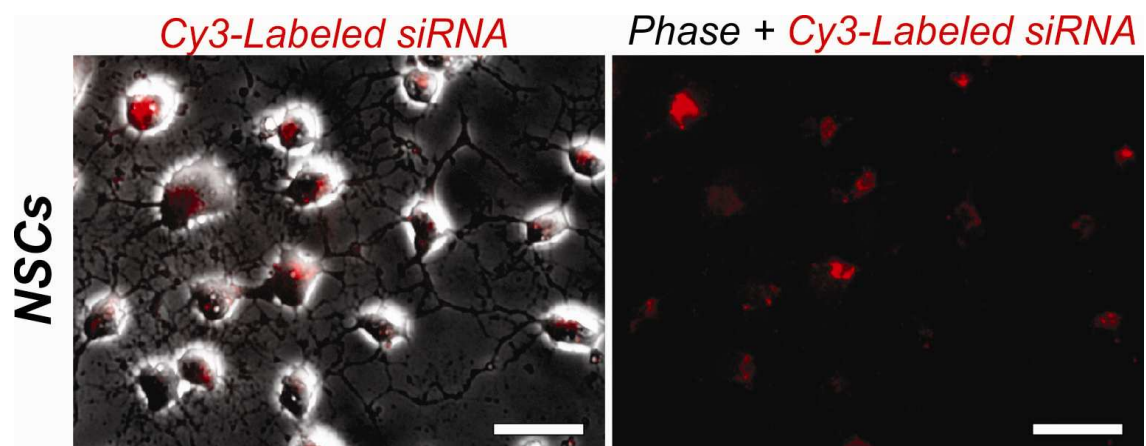


Figure S2. Dye-labeled siRNA uptake in NSC. Fluorescence and phase images depicting the cellular uptake of Silencer® negative control Cy3-labeled siRNA complexed with DexAM in NSCs. Scale bars: 20 μm.

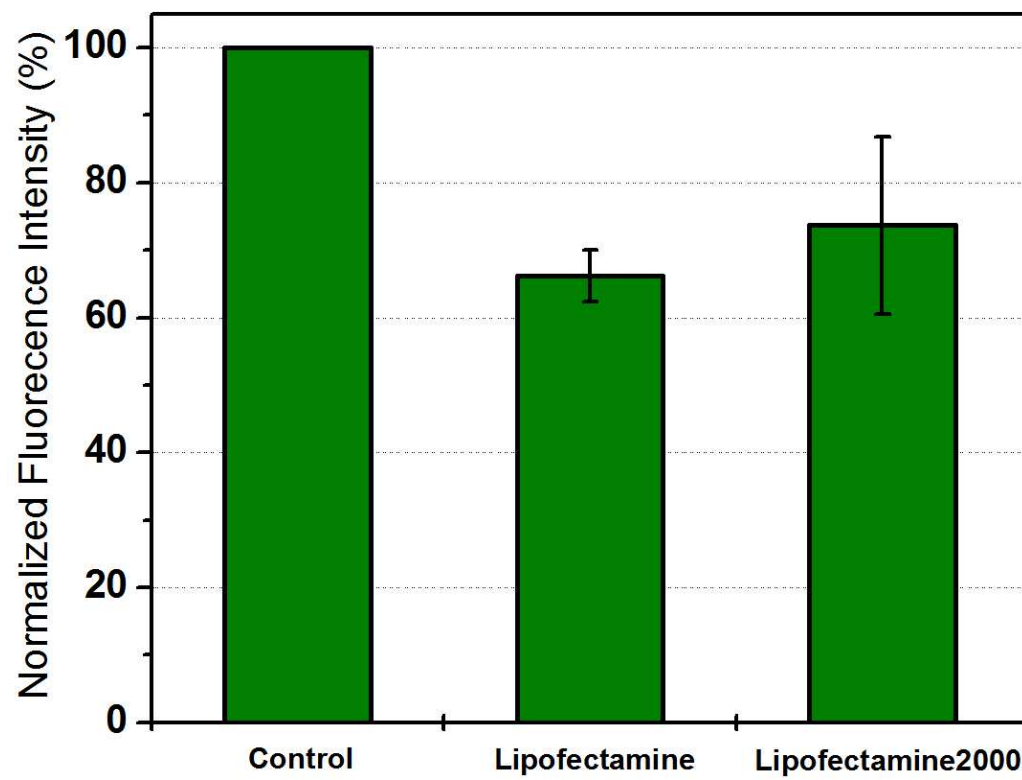


Figure S3. Quantitative comparison of the percentage of GFP knockdown at 96 hrs in NSCs using commercial transfection reagents at recommended doses to deliver GFP siRNA.

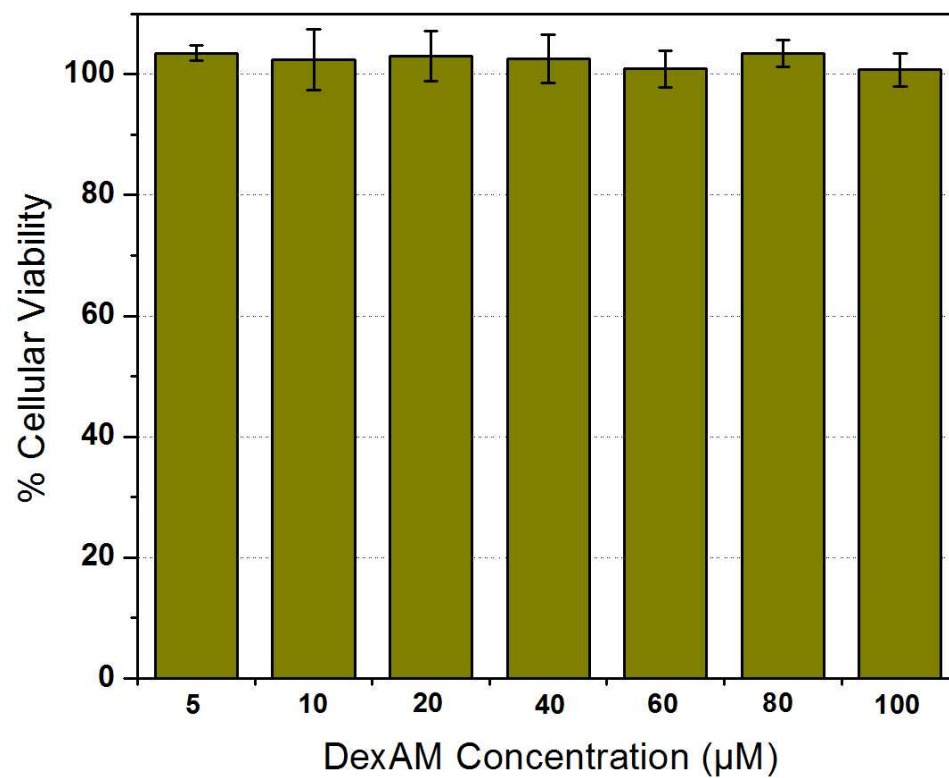


Figure S4. Cell viability of NSCs treated with DexAM. The percentage of viable cells was estimated using MTS assay following incubation of cells with the DexAM-siRNA complexes (100 pmol siRNA) for 96 h. The data was obtained as absorbance of water-soluble formazan at 490 nm, following incubation with only DexAMs for 96 h. The fluorescence for treated samples was normalized to that of untreated controls.

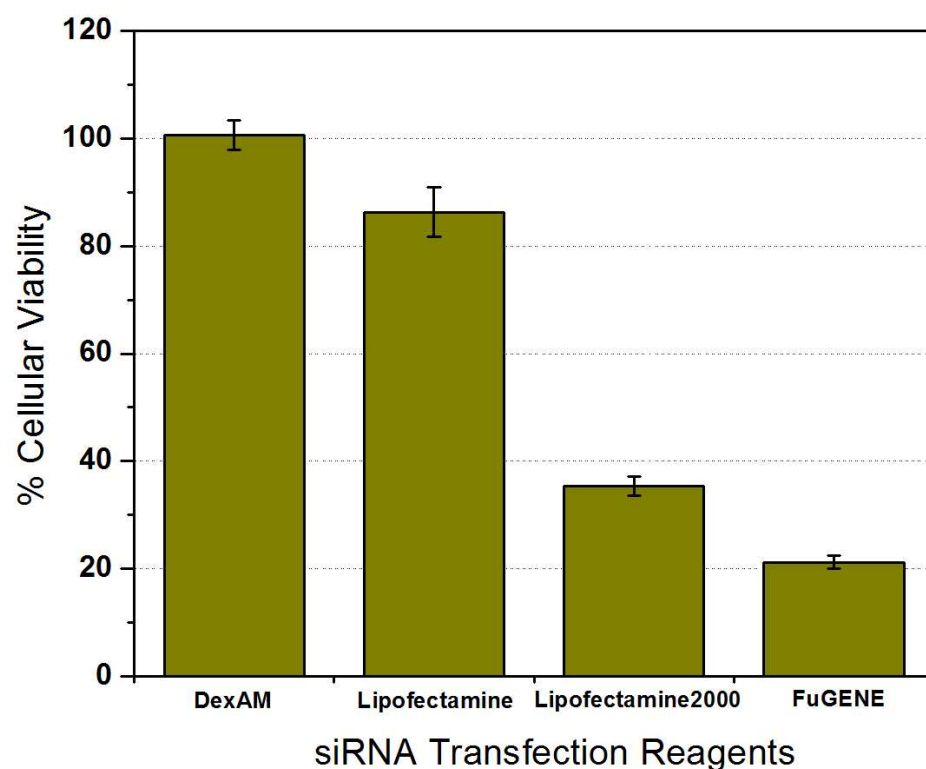


Figure S5. Comparison of NSC viability using commercially available transfection reagents. The percentage of viable cells was estimated after incubation of cells with the recommended concentration of various commercially available transfection reagents (Lipofectamine, Lipofectamine2000, FuGENE) complexed with 100 pmol siRNA for 96 h using MTS assay. The data was obtained as absorbance of water-soluble formazan at 490 nm, following incubation with only DexAMs for 96 h. The fluorescence for treated samples was normalized to that of untreated controls.

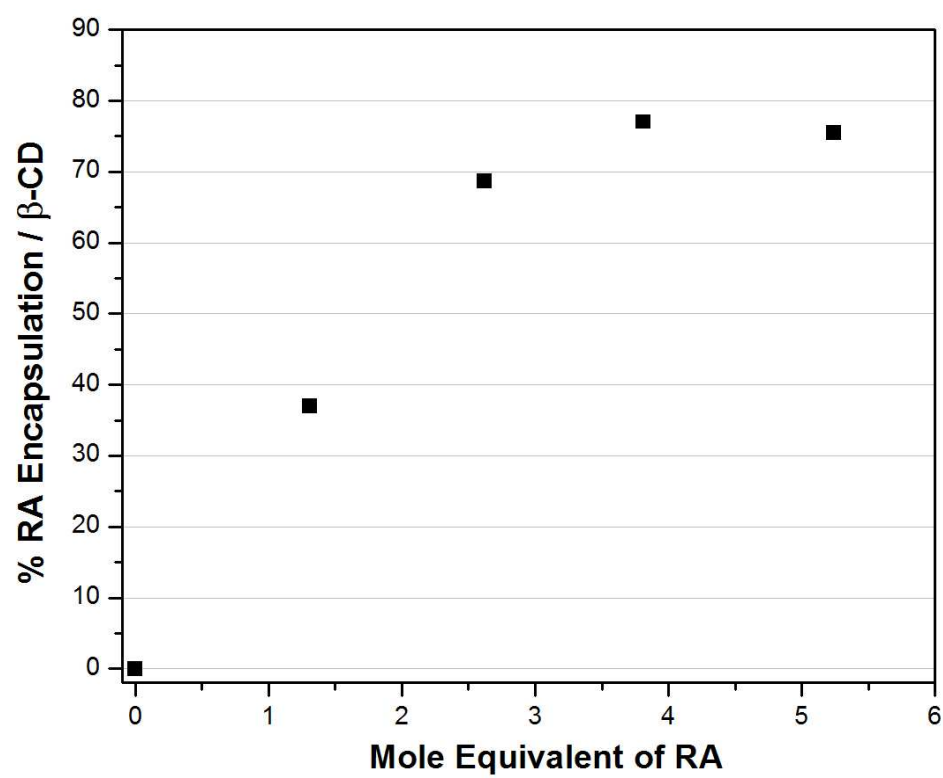


Figure S6. Percentage of RA encapsulation per β -CD on the DexAM.

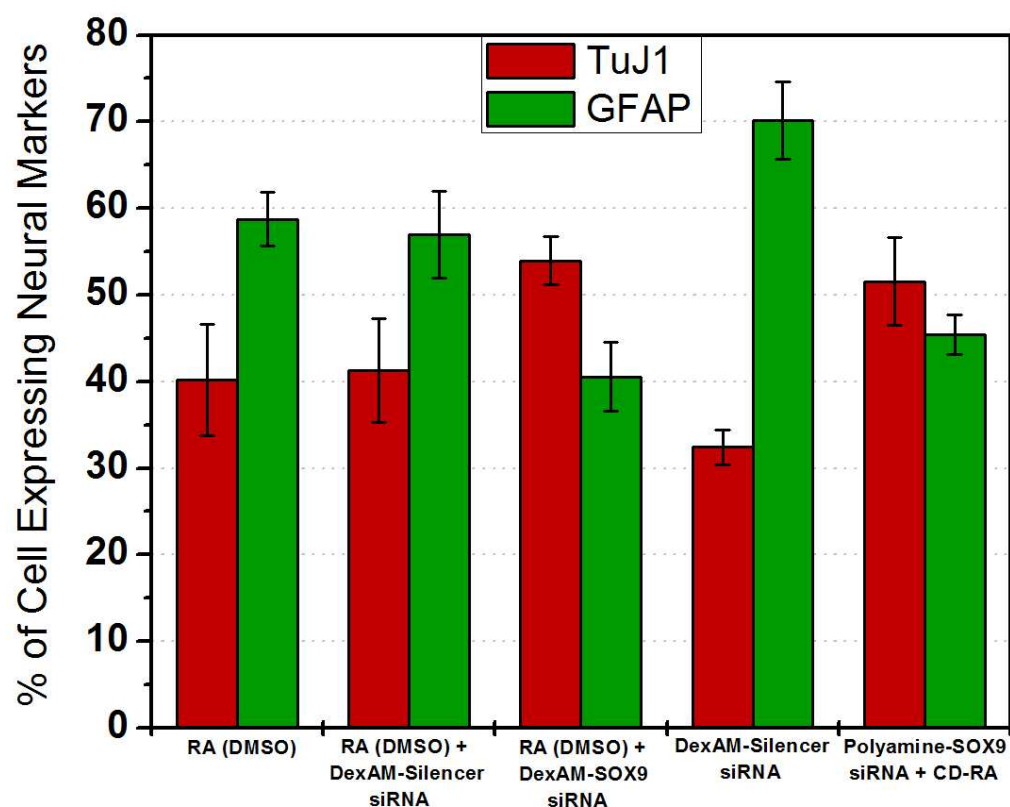


Figure S7. Quantitative comparison of NSC differentiation for various control experiments. Percentage of cells expressing TuJ1 and GFAP at 6 days was calculated for NSCs treated with different combinations of 2 μ M RA in DMSO (0.1% DMSO), DexAM complexes with negative control siRNA (Silencer® siRNA, Ambion), DexAM complexes with SOX9 siRNA, β -CD complexed with RA (CD-RA) and the polyamine (DexAM without β -CD) complexed with SOX9 siRNA.